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## Can Antibiotics from Recently Discovered Marine Actinobacteria Slow the Tide of Antibiotic Resistance?

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CAN ANTIBIOTICS FROM RECENTLY DISCOVERED MARINE  
ACTINOBACTERIA SLOW THE TIDE OF ANTIBIOTIC RESISTANCE?

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

By

LORRAINE SUSAN TANGEMAN  
B.S., Wright State University, 1998

2013  
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

July 10, 2013

I HEREBY RECOMMEND THAT THE THESIS PREPARED  
UNDER MY SUPERVISION BY Lorraine Susan Tangeman  
ENTITLED Can Antibiotics From Recently Discovered Marine  
Actinobacteria Slow The Tide Of Antibiotic Resistance? BE  
ACCEPTED IN PARTIAL FULFILLMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF Master of Science

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## ABSTRACT

Tangeman, Lorraine Susan. M.S., Department of Biological Sciences, Wright State University, 2013. Can Antibiotics From Recently Discovered Marine Actinobacteria Slow The Tide of Antibiotic Resistance?

Actinobacteria, one phylum of gram positive bacteria, are found throughout all the environments on earth. Actinobacteria have long been studied for the benefits they provide, both to their environment and to humans, and have a great capacity for adaptation and evolution. They decompose organic matter, replenishing nutrients into the soil, and as such are important members of the food chain. Humans benefit from the exploitation of Actinobacterial metabolites as antimicrobial drugs. These antimicrobials have been effectively utilized for decades in the fight against infectious disease. Despite the success of this drug arsenal we are now in the midst of an epidemic of multidrug-resistant superbugs that render established drugs ineffective. In order to find new antimicrobial drugs, researchers have turned to the recent discovery of several new species of marine Actinobacteria and analyzed their metabolites for antimicrobial activity. Several metabolites were effective in vitro, and may lead to the development of marketable pharmaceuticals.

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## I. INTRODUCTION

For the past 70 years, we have lived in a period when humans believed infectious disease was able to be conquered. In 1900, infectious diseases claimed the lives of approximately 1 in 100 Americans, and by 2000, that number was reduced to 1 in 300 (Black, 2012). This change is due in part to the discovery and subsequent widespread administration of antimicrobial drugs. These drugs, called antibiotics, have been considered miracle drugs and have contributed to the increase in life expectancy of Americans from 40 years in 1850 to nearly 80 years today (Black, 2012). A few antibiotics are synthetic antibiotics and are completely man-made. Some are semisynthetic antibiotics which are natural antibiotics that have been chemically altered by humans to make them more effective and/or able to withstand microbial resistance mechanisms.

Most antibiotics are natural substances, produced through metabolic processes of bacteria in the genera *Streptomyces* and *Bacillus* (phylum Actinobacteria) or from fungi in the genera *Penicillium* and *Cephalosporium*. These substances are called secondary metabolites. Actinobacteria are best known for their production of secondary metabolites,



which are secreted cellular molecules that have been exploited by humans as pharmaceutical chemicals. Many antibiotics, anti-cancer drugs, anti-viral drugs, and other medicines have been derived from Actinobacteria. The genes for these metabolites are induced when the culture encounters nutrient depletion and/or growth rate decrease, and are understood to play a role in regulating the growth of competitors for space and nutrients. These metabolites may serve as quorum sensing molecules, fostering communication between species in an ecosystem, particularly biofilms (Demain, 1998). Within the complex community of marine invertebrate symbiosis, the secondary metabolites may enable the bacteria to provide nutrition and chemical protection in exchange for habitat (Lam, 2006; Ward, Bora, 2006).

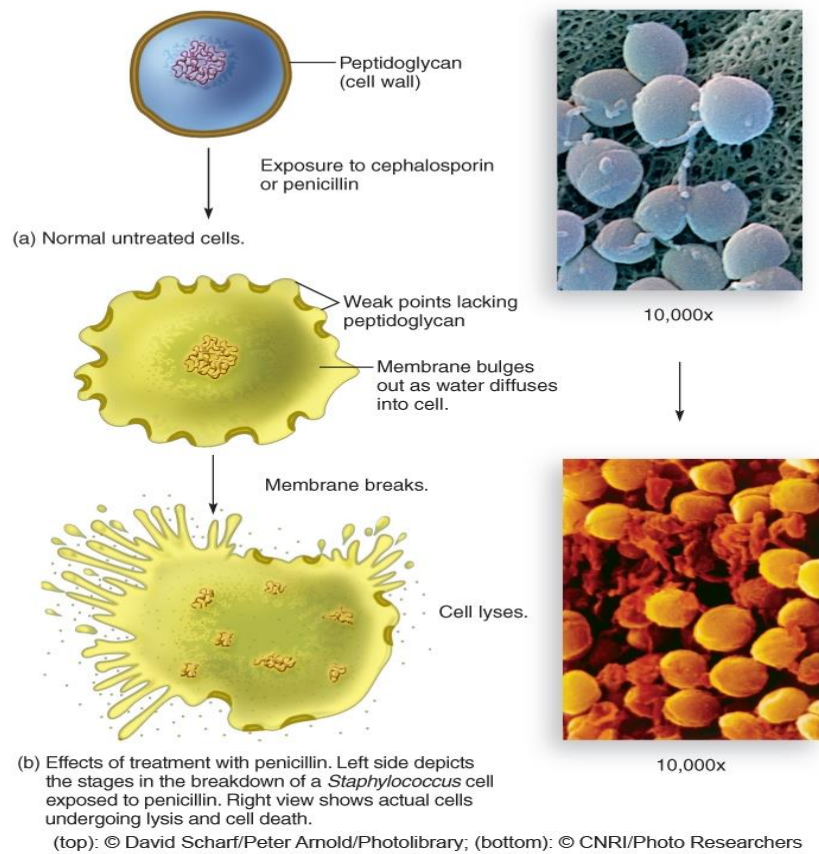
Historically, any Actinobacteria found in the oceans were believed to originate as spores from terrestrial bacteria washed into the sea (Goodfellow, Haynes, 1984). Over the past several years, however, a significant body of research has demonstrated that these microbes reside within life forms, the seabed, and the seawater, and require seawater for growth (Bull, et al., 2005). Some of these marine isolates are new strains of known genera, but phylogenetic analysis of other isolates has led to the discovery of two new genera, *Salinispora*, (Jensen, et al., 2005) and *Marinispora* (Kwon, et al., 2006). These unique isolates have been found in all levels of the oceanic ecosystem, from surface water to sub-floor sediments, and in symbiotic relationships with marine invertebrates (Ward, Bora, 2006).

## II. CLASSES OF ANTIBIOTICS

Antibiotics are classified according to their mode of action. One class includes antibiotics that interfere with cell wall biosynthesis. Bacterial cell walls are made of peptidoglycan, a rigid meshwork of chains of repeating sugar units cross-linked with short peptides. Gram-positive organisms have a thick peptidoglycan layer. Gram-negative organisms, in contrast, have a thin layer surrounded by an outer membrane containing lipopolysaccharide. Embedded within the outer membrane are protein channels called porins. By closing these porins, the outer membrane affords an extra layer of protection by limiting entry of antibiotics. The classes of antibiotics which act on the cell wall include glycopeptides and  $\beta$ -lactams. The chemical structure known as a  $\beta$ -lactam ring binds to and inactivates peptidoglycan transpeptidase, the enzyme responsible for cross-linking the peptides between the sugar backbone chains (Salyers, Whitt, 2002). The result is a weakened cell wall which is then subject to osmotic lysis (Figure 1). Penicillins, cephalosporins, monobactams, and carbapenems make up the  $\beta$ -lactams. The glycopeptides vancomycin and teicoplanin also disrupt the integrity of the peptidoglycan cell wall by preventing transpeptidation. These molecules bind to the end of the free peptide chains before they can be cross-linked. Due to their large size, the

glycopeptides have a narrow spectrum of activity; they cannot penetrate the porins of the gram-negative outer membrane.

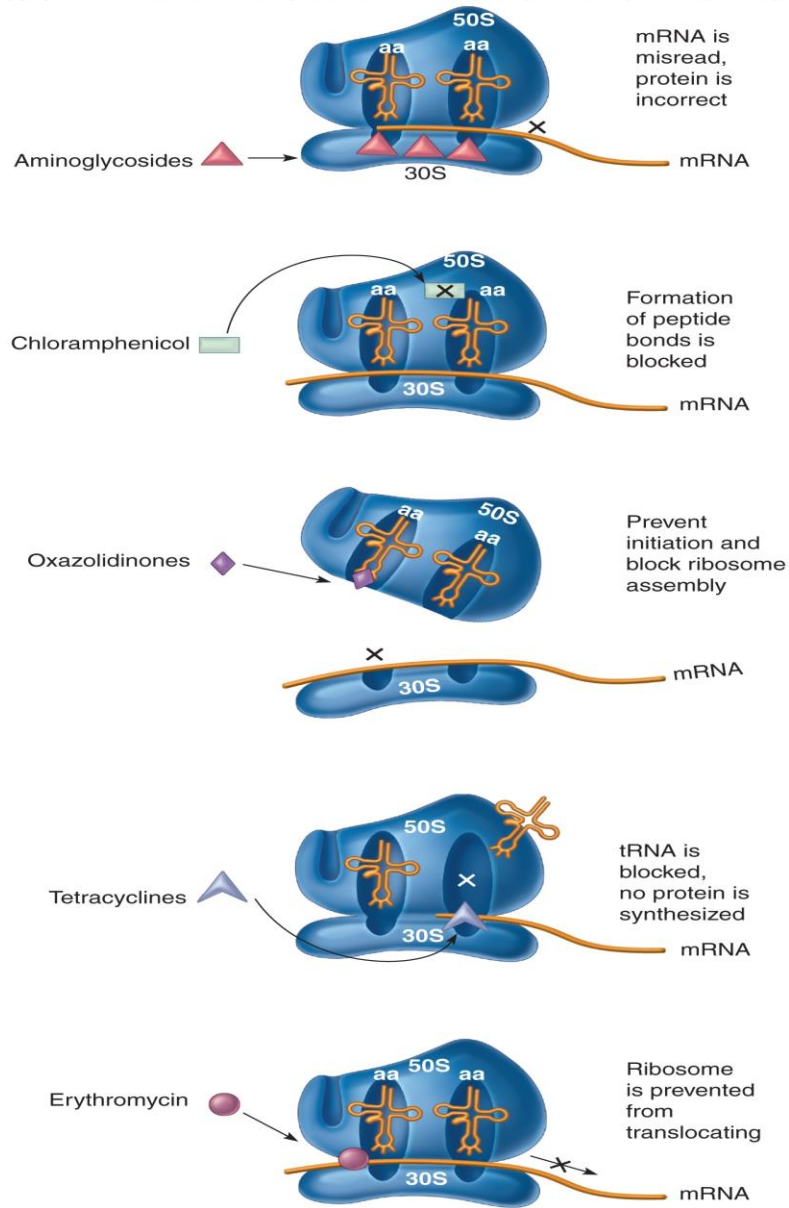
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**Figure 1. Effect of beta-lactam antibiotics on bacterial cell walls**  
Adapted from Cowan, 2012

A large class of antibiotics includes drugs that block protein synthesis by binding to ribosomes. Ribosomes are made of ribosomal RNA (rRNA) and protein, in the form of two subunits. In bacteria, the small subunit (30s) consists of approximately twenty proteins and a 16s rRNA of 1500 nucleotides. The large subunit (50s) consists of approximately 30 proteins and a 23s rRNA of 2900 nucleotides. At the surface of the large subunit are the A, P, and E codon sites. It is to the A site that the aminoacyl tRNA's are ushered by the chaperone protein EF-Tu (Walsh, 2003). Within this structure are many potential antibiotic binding sites which will interrupt protein synthesis (Figure 2). Aminoglycoside antibiotics, represented by streptomycin, kanamycin, and gentamicin, also bind to the 30s subunit, but do so in a way that prevents a good fit between the subunits and the mRNA, causing a misread of the mRNA. Chloramphenicol binds to the 50s subunit so that peptide bonds cannot form between incoming amino acids. Another group, the oxazolidinones, bind in the P site in the peptidyltransferase center, blocking the first step of peptide bond formation (Walsh, 2003). The tetracycline antibiotics bind to the 30s subunit to block the binding of aminoacyl tRNA's at the A site. The macrolides, which include erythromycin, bind and block the exit tunnel for the elongating peptide chain. The macrolide structural elements make up to seven bonds with the 23s rRNA of the large subunit.

Another class of antibiotics interferes with cell membranes. These antibiotics interact with membrane phospholipids and either cause a disruption of metabolic processes or



**Figure 2. Antibiotics that act on the prokaryotic ribosome**  
Adapted from Cowan, 2012

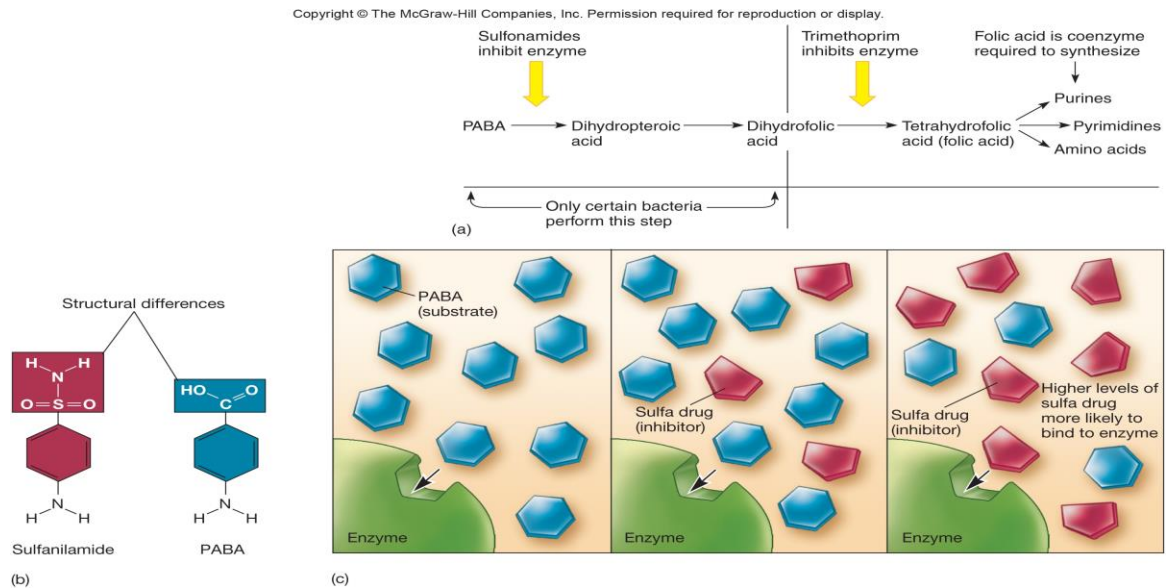
membrane lysis. Some antibiotics in this class, such as polymixin, tend to be non-selective and can damage human membranes as well. Daptomycin, however, is not as toxic and is effective against gram-positive organisms (Cowan, 2012).

The next major class of antibiotics acts by blocking DNA replication and repair. These include quinolones, fluoroquinolones, and novobiocin. These antibiotics target the enzymes that control the coiling and uncoiling of DNA during the replication process. Quinolones specifically target topoisomerase II, an enzyme that makes temporary cuts in DNA to relieve supercoiling during replication. When the enzyme cuts the DNA, it binds to and holds the free ends. Quinolones act by binding to this complex, preventing re-ligation. This halts the replication forks, signaling cell death (Walsh, 2003).

Rifamycin antibiotics are RNA polymerase inhibitors. They bind in the DNA/RNA tunnel, blocking the elongation of the mRNA chain at the di- or tri-nucleotide stage. These antibiotics are currently used primarily for the treatment of tuberculosis (Walsh, 2003).

The final class of antibiotics includes drugs that target tetrahydrofolate (folic acid) synthesis. These act as competitive inhibitors for key enzymes in the synthesis pathway (Figure 3). Tetrahydrofolate is necessary for proper formation of nucleotides; it carries and donates carbon atoms in the nucleotide synthesis reaction (Garrett, Grisham, 2005).

Without tetrahydrofolate, a cell cannot replicate DNA and cannot divide. One of the first antibiotics available to the general public, sulfonamide (sulfa), is in this class.



**Figure 3 Tetrahydrofolate inhibition**  
Adapted from Cowan, 2012

### III. STATE OF ANTIBIOTIC RESISTANCE

In recent years, infectious disease has been on the rise, and by 2007, was back in the top two causes of death in the world, and in the top three in the United States (Spellberg, et al., 2008). An aging population and growing numbers of immunocompromised individuals, both with weakened immune systems, contribute to this increase (Boucher, 2010). Another is a change in human practices, such as international travel, the widespread use of air conditioning, or overcrowding in public housing areas such as prisons, slums, or hospitals. These changes bring individuals into contact with microbes that never before had the chance to cause widespread disease. The largest factor, though, has been the microbes themselves. As the Infectious Diseases Society of America states, “It is absurd to believe that we could ever claim victory in a war against organisms that outnumber us by a factor of  $10^{22}$ , that outweigh us by a factor of  $10^8$ , that have existed for 1000 times longer than our species, and that can undergo as many as 500,000 generations during 1 of our generations” (Spellberg, et al., 2008). Very soon after a new drug is approved for use, reports of resistance arise. Most resistance is attributed to overuse of the drug, in both humans and livestock. Because of huge populations and rapid generation times, microbes can quickly mutate to accommodate a change in their



environment. They also easily acquire new genes for virulence factors or resistance mechanisms from neighboring microbes through lateral gene transfer, and many of those genes originate from the same organisms that created the antibiotic as a self-survival mechanism (Hopwood, 2007). Resistant organisms are especially prevalent in hospitals, where exposure to antibiotics is almost constant. Resistance can arise quickly due to antibiotic selective pressure.

Mechanisms of resistance take many forms (Figure 4). The first, used by gram negative organisms, is limiting access of the antibiotic to the cell by restricting entry through outer membrane porins. Another is the enzymatic destruction or modification of the antibiotic.  $\beta$ -lactamase is an enzyme that will hydrolyze penicillin. Following the development of resistance to  $\beta$ -lactam antibiotics, the antibiotics were then modified to include either a  $\beta$ -lactamase inhibitor like clavulanate, or bulky side groups which blocked the enzyme from reaching its active site. Aminoglycoside antibiotics are rendered inactive when a modifying enzyme covalently changes the hydrogen binding site through acetylation, phosphorylation, or adenylation (Shahid, Malik, 2005).

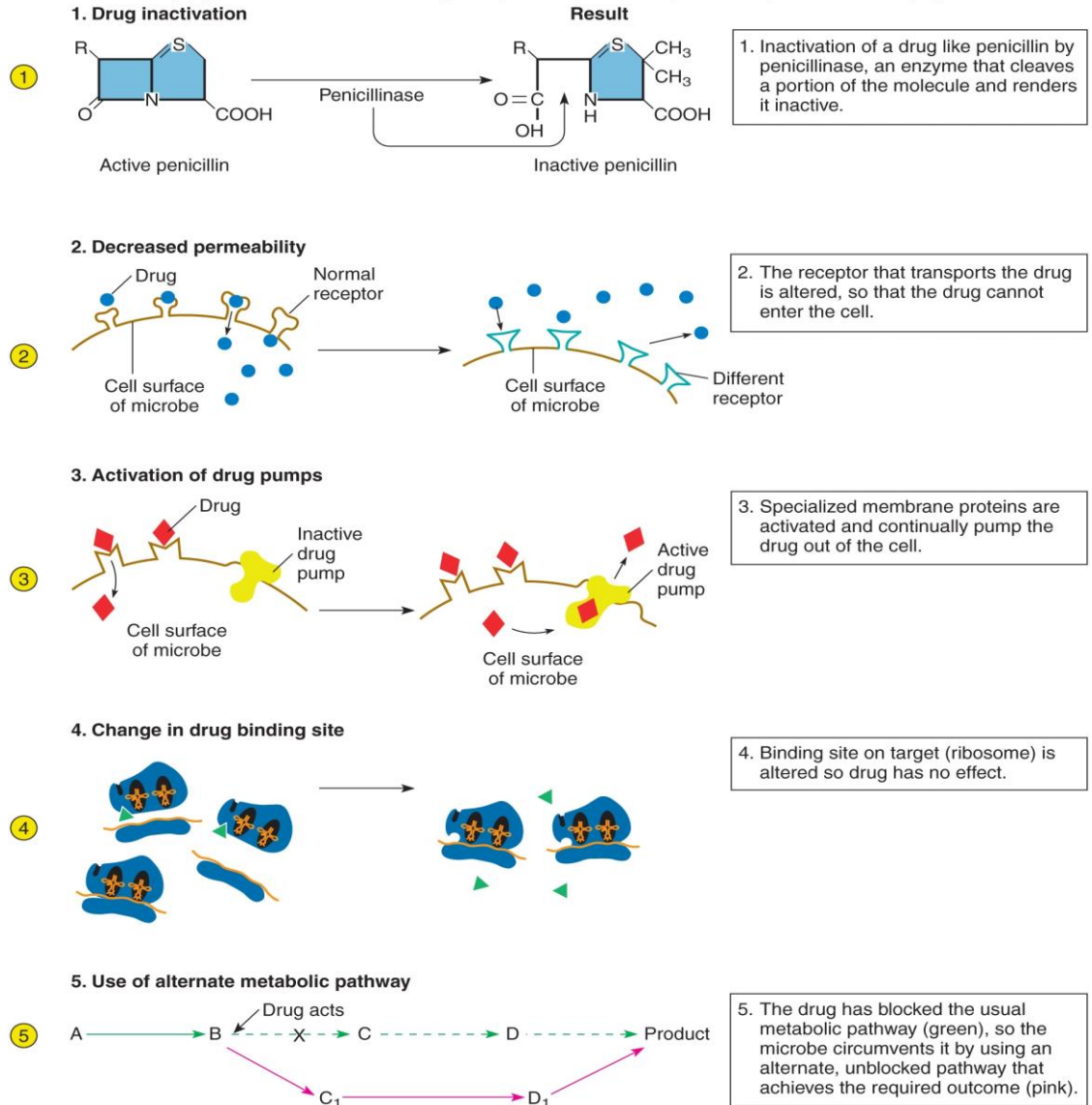
Many organisms use active efflux to remove an antibiotic from the cell. Some of these pumps have a narrow specificity, pumping only one antibiotic, others are broad spectrum. These broad spectrum pumps are often called multidrug resistant (MDR) pumps, and protect the cell from many drugs and other harmful chemicals. The pumps have

hydrophobic transmembrane and hydrophilic cytoplasmic domains and act in a manner similar to the proton motive force. They may also act to export virulence factors such as enzymes and toxins. Screens for pump genes may be used to evaluate culture isolates for potential resistance (Ping, et al., 2007).

Another resistance mechanism is to modify or replace an antibiotic's target. The methicillin resistance gene (*mecA*) results in a new penicillin binding protein, PBP2A (Memmi, et al., 2008), which has a lower binding affinity. Resistance to macrolide antibiotics is achieved by methylation of the peptidyl transferase cavity (Bowers, et al., 2012). Widespread use of a vancomycin-like antibiotic in cattle feed and an increase in the treatment of opportunistic enterococci infections has led to increased vancomycin resistance. This is brought about by a cassette of five genes (*vanR*, *vanS*, *vanH*, *vanX*, and *vanA* or *B*) (Perichon, Courvalin, 2009) that is plasmid borne. These genes change the terminal peptide sequence of peptidoglycan cross links by substituting a lactate molecule for the terminal amino acid alanine. Vancomycin cannot bind, and the peptidoglycan can still cross link.

The final mechanism of resistance is to change metabolic pathways. Microbes gain resistance to sulfonamide/trimethoprim by altering the folic acid synthesis pathway. This most commonly occurs via a mutation in the gene for either ADC synthase, which confers sulfa resistance, or dihydrofolate reductase, conferring trimethoprim

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**Figure 4. Mechanisms of Acquired Drug Resistance**  
Adapted from Cowan, 2012

resistance (Matthews, et al., 1984). These modifications render the microbe resistant, but still allow for cellular function.

In 2004, the Infectious Disease Society of America (IDSA) issued a policy report titled “Bad Bugs, No Drugs: As Antibiotic R&D Stagnates, a Public Health Crisis Brews”, with the intent to raise awareness for the need to renew antibiotic development. (Boucher, 2004) In late 2007, the IDSA followed with an update which declared “We are in the midst of an emerging crisis of antibiotic resistance for microbial pathogens in the United States and throughout the world” (Spellberg, et al., 2008). This update highlighted a global pandemic of antibiotic resistance, the lack of antibiotic discovery, and a need for strategies to address antibiotic use. A few months later, the National Institute of Allergy and Infectious Disease (NIAID) identified groups of “superbugs” that are of special concern (Peters, et al., 2008). The first are lung pathogens showing high rates of resistance: *Streptococcus pneumoniae* and multidrug resistant (MDR) and extensively drug resistant (XDR) *Mycobacterium tuberculosis*.

*Streptococcus pneumoniae*, the leading cause of community-acquired pneumonia, otitis media and meningitis, has become multidrug resistant after developing resistance to macrolide antibiotics such as erythromycin. Erythromycin had been the treatment of choice since many *S. pneumoniae* are now resistant to penicillin (Hidalgo, et al., 2003). The genes that confer macrolide resistance, *mef E* and *erm B*, are carried by a transposon

that also carries tetracycline resistance. The *mefE* gene codes for an efflux pump, while *ermB* codes for a methyltransferase resulting in methylation of the ribosomal target (Bowers, et al., 2012). In cases of multidrug resistance, vancomycin has frequently been the treatment of choice. Recently, vancomycin treatment failure has been increasing, even though the organism shows susceptibility in drug sensitivity testing. These treatment failures are due to a phenomenon called vanco-tolerance. Tolerance is defined as the ability to survive under antibiotic selective pressure, without evidence of growth (Olivares, et al., 2011). The clinical definition of tolerance is a minimum bactericidal concentration (MBC) that is 32X the minimum inhibitory concentration (MIC) (Safdar, Rolston, 2006). The first report of tolerance in *S. pneumoniae* was in 1999 (Novak, et al., 1999). Tolerance is considered the precursor to resistance, which would eliminate one of the important antibiotics for multi-drug resistant *S. pneumoniae* (Hidalgo, et al., 2003). Compounding the health threat, these resistance mechanisms are found in serotypes that are frequently not included in the heptavalent pneumococcal vaccine. A newly approved 13-valent vaccine should change the prevalence of resistance (Bowers, et al., 2012).

Tuberculosis (TB) is one of the world's deadliest diseases. It is believed that one-third of the world's population is infected with *Mycobacterium tuberculosis*. Nine million new cases and 1.5 million deaths were reported in 2011 (<http://www.cdc.gov/tb/statistics/default/htm>, [http://who.int/tb/publications/global\\_report/gtbr12\\_executivesummary.pdf](http://who.int/tb/publications/global_report/gtbr12_executivesummary.pdf)). Because of the structure and metabolism of this organism, treatment requires multiple

drugs that must be given for six months or longer. Latent infections, in which the *Mycobacterium* are present in the body, but the patient shows no signs or symptoms, are treated with any of three first line drugs, isoniazid, rifampin, and rifapentine, either singly or in various combinations for two to eight months (<http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5211a1.htm>). TB disease, in which the bacteria are actively growing, is treated more aggressively. The preferred regimen uses four first line drugs- isoniazid, rifampin, ethambutol, and pyrazinamide taken daily for eight weeks, followed by several options of continuation treatments for another four to seven months (<http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5211a1.htm>). Because of the rigorous nature and the costs involved, this regimen is not always followed, and has led to the development of antibiotic resistance. Tuberculosis resistance arises, not from lateral gene transfer, but by normal bacterial mutations that change the antibiotic's target by adjusting functional enzymes, changing cell wall synthesis, or ribosomal proteins (Feuerriegel, et al., 2012). Improper use of antibiotics can cause further mutations due to antibiotic selective pressure. Two levels of resistance have been defined by the World Health Organization (WHO). Multidrug resistance (MDR-TB) is defined as resistance to both isoniazid and rifampin. Extensively drug resistant tuberculosis (XDR-TB) has resistance to both isoniazid and rifampin, as well as a fluoroquinolone and one of the three second-line drugs, amikacin, kanamycin, and capreomycin. Of greater concern, although considered inevitable, are the recent reports of what is being called totally drug resistant tuberculosis (TDR-TB), although neither the CDC nor the WHO have accepted that

designation. Three instances of TDR-TB have been reported: in Italy in 2007 (Migliori, et al., 2007), in Iran in 2009 (Velayati, et al., 2009), and most recently, twelve individuals in Mumbai, India earlier last year (Udwadia, et al., 2012). The Indian strain did not respond to treatment or susceptibility testing for twelve different antibiotics. So far three of the twelve individuals have died. The remaining nine are “walking the streets”, spreading the disease, because of lack of hospital beds and costs of isolation. These cases may just be scratching the surface, because the lack of medical infrastructure in India results in many undiagnosed cases (Loewenberg, 2012).

Enteric anaerobes, as opportunistic pathogens, are another of the “superbug” groups. These organisms are normal intestinal biota but are pathogenic when introduced into an unusual body site or when there is a disturbance in the gut environment. Notable microbes within this group are *Clostridium difficile* and *Bacteroides fragilis*. *Clostridium difficile* is very resistant to many broad spectrum antibiotics, especially penicillins, cephalosporins, and clindamycin. These antibiotics are administered for some other common infection, with a side effect of eradicating much of the normal intestinal biota. *C. difficile* can then proliferate, causing a superinfection, leading to potentially severe gastrointestinal distress. Current treatment for *C. difficile* infections is either metronidazole or vancocin (oral vancomycin), but it is particularly difficult to cure due to its spore-forming capability (Louie, et al., 2011). A new hypervirulent strain has emerged, called PCR ribotype 027 (Stabler, et al., 2009). This strain is associated with

increased toxin production, bringing more severe diarrhea, a higher mortality rate, and a greater chance of recurrence.

*Bacteroides fragilis* is common in post-operative infections, particularly abdominal surgeries, abdominal perforations, trauma infections, and blood infections (Trevino, et al., 2012). Results from five years of surveillance show a gradual overall increase in resistance, but it varies from isolate to isolate (Snydman, et al., 2012). Resistance is mediated by a resistance-nodulation division (RND) type multidrug efflux pump, which is capable of removing multiple classes of antibiotics (Wexler, 2012). A recent report details resistance to carbapenems (imipenem) due to the production of a metallo-beta lactamase (Trevino, et al., 2012).

The last of the “superbugs” is a group of pathogens responsible for most nosocomial infections; it has been dubbed the ESKAPE group (Rice, 2008). ESKAPE is an acronym for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.

*E. faecium* is gram positive normal biota of the human gastrointestinal tract and has become increasingly resistant to most antimicrobials, including vancomycin. Resistance in *E. faecium* arises from both mutations and lateral gene transfer from other gut biota (Brisson-Noel, et al., 1990). The plasmid-borne *vanA* codes for a ligase that changes the



terminal peptide sequence of vancomycin's peptidoglycan target (Perichon, Courvalin, 2009). In some areas of the world, the rates of vancomycin resistance is as high as 60% (Boucher, et al., 2009). Individuals most affected are those in intensive care and the immunocompromised, and their infections are most likely to be post-operative infections, bloodstream, or urinary tract infections arising from inline catheterization. Catheters and other invasive medical devices are prone to biofilm formation. A functional biofilm enhances colonization, provides a matrix for microbial growth through the production of an exopolysaccharide (Gordon, Wareham, 2010), and protects the organisms from adverse environmental pressures. The presence of biofilms reduces the efficacy of both cleansing techniques and drug regimens, making them harder to remove. Both vancomycin and linezolid are unable to penetrate biofilms formed by *E. faecium* (Bayston, et al., 2012). Drug resistant *E. faecium* is disseminating throughout the environment, as it has been isolated from both wild and domesticated animals, including cats (Ghosh, et al., 2012), poultry (Tremblay, et al., 2011; Sapkota, et al., 2011), fish (Araujo, et al., 2011), birds (daSilva, et al., 2011), wolf (Goncalves, et al., 2011), and fox (Radhouani, et al., 2011).

*Staphylococcus aureus* has been a cause of significant morbidity and mortality for centuries, despite huge advances in medical care. In fact, as our knowledge and technology increases, the microbe adapts and infections become more complex (Boucher, Corey, 2008). It is mostly implicated in skin and wound infections but has the ability to

move deeper into tissues and cause multiple organ infections and bloodstream infections. Like the enterococci, it commonly forms biofilms that are resistant to eradication (Bayston, et al., 2012). Methicillin resistant *Staphylococcus aureus* (MRSA) has become a household word in the United States. While once almost exclusively a nosocomial infection (HA-MRSA), community associated (CA-MRSA), or cases arising without previous history of hospitalization, is becoming more common (Memmi, et al., 2008). MRSA has also been isolated from farm animals (Gharsa, et al., 2012) and found in dairy milk (Haran, et al., 2012). On the horizon are vancomycin intermediate *Staphylococcus aureus* (VISA) and vancomycin resistant *Staphylococcus aureus* (VRSA). VRSA is believed to be derived by lateral gene transfer of the resistance transposon Tn1546 carrying the *vanA* resistance gene cluster from *Enterococcus* sp. (Perichon, Courvalin, 2009). There have been thirteen confirmed cases of VRSA in the United States, ten of which were confirmed *vanA* genotype, and additional cases have been reported throughout the world. The last case of VRSA in the United States was in July 2012. VISA has been on the rise since 2007, rising from 37 cases in 2007 to 130 reported in 2012. Thus far in 2013, 51 cases have been reported (<http://www.cdc.gov/mmwr/pdf/wk/mm6218md.pdf> ). A new category of vancomycin resistance is heterogeneous VISA (hVISA), which is a clinical “mixture” of a susceptible strain (MIC <4 mg/mL), with a subpopulation of intermediate organisms with a MIC>8 mg/mL. This heterogeneous category is considered the precursor to vancomycin intermediate strains (Conly, Johnston, 2002). Intermediate resistance occurs by a

different mechanism than lateral gene transfer. Organisms have a thickened cell wall, which is believed to prevent the vancomycin from penetrating deeply enough to compromise the integrity of the peptidoglycan (Conly, Johnston, 2002). In order to identify possible genetic mutations that would lead to the thickened cell wall, Hafer, et al. screened the genome of many VISA isolates for possible aberrations and found point mutations in many gene loci (Hafer, et al., 2012). The greater number of mutated genes correlated with a higher level of resistance of the organism, but it is not yet known how the mutations lead to cell wall thickening or vancomycin resistance. It has been suggested that this intermediate pathway will lead to a resistant phenotype without the acquisition of *vanA* (Tiwari, Sen, 2006).

The remaining four ESKAPE organisms are gram-negative opportunistic pathogens. *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are ubiquitous environmental organisms (Pakyz, et al., 2009; Telang, et al., 2011) while *Klebsiella pneumoniae* and other members of Enterobacteriaceae are normal biota of the human gastrointestinal tract. These organisms are transmitted easily from human to human and through every environment, and are effective biofilm producers (Gordon, Wareham, 2010). This ubiquitous nature makes them very difficult to eradicate from the medical setting (Telang, et al., 2011). The most common infection sites include the respiratory tract, particularly associated with ventilators, the urinary tract usually associated with catheters, and wound, bloodstream, and burn infections. Besides innate resistance due to outer

membrane porin modifications, these bacteria have acquired resistance mechanisms for nearly every antibiotic available, including drug target mutations, aminoglycoside modifying enzymes (Shahid, Malik, 2005), and efflux drug pumps (Gordon, Wareham, 2010; Sonnet, et al., 2012). Since 2000, extended spectrum beta-lactamases have increased resistance to all beta lactam antibiotics, including all cephalosporins, leaving carbapenems as the antibiotic of last resort. As could be predicted, carbapenem resistance has since been on the rise worldwide due to the acquisition of carbapenemase genes (Nordmann, Naas, et al., 2011), but of greatest concern is the appearance of a new resistance gene. In 2007, a Swedish patient who had been hospitalized in India was diagnosed with a new strain of multidrug resistant *K. pneumoniae* from a urinary tract infection that was resistant to carbapenems. This new resistance gene was named New Delhi metallo-beta-lactamase 1 (NDM-1)( Yong, et al., 2009). NDM-1 has been identified on several different large plasmids, each one also containing a high number of resistance genes for other antibiotic classes including cephalosporinase genes, aminoglycoside, macrolide, rifampin, and sulfonamide resistance genes (Bonnin, et al., 2012). It has spread from *Klebsiella* and has been isolated in *Acinetobacter* (Nordmann, Poirel, et al., 2011), *E. coli* (Mulvey, et al., 2011; Bonnin, et al., 2012), and *Salmonella* (Moellering, 2010). The incidence of carbapenemases has increased world-wide, including frequent reports of community acquired cases. Increasingly worrisome are recent reports of NDM-1 being found in the public tap water supplies and in environmental water, such as streams, ponds, rivulets, etc., in India (Walsh, et al., 2011)

and the identification of NDM-1 isolates in food animals in China (Wang, et al., 2012).

A new variant, NDM-2, with an identical spectrum of resistance, has also been described from a child hospitalized in Cairo, Egypt, and moved to Frankfurt, Germany (Kaase, et al., 2011). Since this first isolation, additional cases have now been reported in other countries in the Middle East (Espinal, et al., 2011; Ghazawi, et al., 2012).

#### IV. ISOLATION OF ANTIMICROBIAL METABOLITES

This acquisition of antibiotic resistance has led researchers on a continuing search for new antimicrobial agents, and the new marine Actinobacteria provide promising novel metabolites that are under investigation as possible antibiotics. All the metabolites discussed in this review were found using a common sampling and testing protocol, and that path from isolation to recovery and identification of antimicrobial metabolites is an arduous task. First, samples are taken from diverse marine habitats. The samples obtained are then cultured in various seawater-based formulations of agar media in different environmental conditions, with the intent to optimize the number of unique isolates. Once each isolated microbe colony is identified using 16s rRNA sequencing techniques, it is subcultured in agar and liquid media that is once again manipulated with various salt and nutrient concentrations and environmental conditions, in order to optimize secondary metabolite production. The generated extracts are collected and purified using high-performance liquid chromatography, and the chemical structure of each is determined using mass spectroscopy and nuclear magnetic resonance spectroscopy. Sometimes the metabolite is identified as an already known compound that may or may not exhibit cytotoxic activity. Any novel metabolite is then tested for

bioactivity against various pathogens, such as MRSA, VRE, *Mycobacterium* spp., and fungi, and cancer cell lines.

There are many limitations to this procedure that result in missing many potential isolates and their metabolites. Marine ecosystems are varied and complex, making a thorough sampling nearly impossible. Despite improvements in culturing techniques, it is estimated that 99% of marine microorganisms are uncultured or unculturable, leaving a vast number of species that could be exploited if isolated (Maldonado, et al., 2005). The use of 16S rRNA identification improves the chance of isolation of novel species.

Optimizing growth conditions for the production of secondary metabolites can also be difficult. As can be expected, it may only generate a fraction of possible metabolites, since the gene for a metabolite may only be induced under very specific conditions. Sequencing technology has revealed that genomes encode far more metabolites than originally thought (Jensen, 2010).

## V. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION

The test to determine a compound's ability to inhibit microorganisms is rather straightforward; the microbes are grown in a culture medium containing the compound in question. This antibiotic susceptibility test is a broth dilution method. The antibiotic is incorporated into a liquid medium in a series of doubling dilutions, typically 16-8-4-2-1-0.5  $\mu\text{g/mL}$ . Each tube is then inoculated with a known quantity of microbes, usually  $5 \times 10^5$ , and allowed to incubate. The lowest concentration of antibiotic that displays no visible growth is the minimum inhibitory concentration, or MIC (Forbes, 2007). The lower the MIC value seen in testing, the more effective the antibiotic is against that microbe. Traditionally, this is determined visually by observing turbidity in test tubes, indicating bacterial growth. This method requires a large quantity of materials, labor, and space to carry out, so it has been modified for use in microdilution testing, which is the most widely accepted method as set by Clinical and Laboratory Standards Institute (CLSI, 2008). The method saves media and labor since the microtiter plate typically includes preset dilutions of usual antibiotics. The plate is still read visually but it has been enhanced by using colored lights and/or reflective surfaces, decreasing the possibility for errors. Automated systems are also available, in which the microbe



inoculum is placed into a “filling tube” which fills a microtiter plate, and the plate is then incubated, while a spectrophotometer measures light transmittance every 15 minutes. No change in transmittance indicates no bacterial growth; reduced transmittance indicates turbidity from microbial growth, meaning resistance to the tested antibiotic.

A new method showing promising results uses real-time polymerase chain reaction, also known as quantitative polymerase chain reaction (Q-PCR), following a short incubation period in media that contains a specific quantity of antibiotic. Q-PCR uses fluorescently labeled nucleotides that can be detected by an optical reader. This is quantitative because the more sequence copies in the original sample, the more PCR copies will be made, resulting in faster detection of fluorescence. If the organism is resistant to the antibiotic, more bacteria will grow during incubation. A larger number of bacteria following the incubation period results in faster detection of a fluorescent signal from the reaction, indicating antibiotic resistance. Original protocols used species-specific 16s rDNA primers for culture-identified pathogens (Rolain, et al., 2004). An updated method uses a single universal 16s rDNA primer against unidentified cells directly isolated from a positive blood culture (Beuving, et al., 2011). The improvement reduces testing time from days to hours, since additional subcultures are not needed.

The test results presented in the following tables, the *in vitro* determination of MIC, is only the first step in the determination of the effectiveness of a potential antibiotic for

human use. Before a new compound can be used, it must undergo *in vivo* tests to determine efficacy, toxicity, and pharmacokinetics, which is the ability to penetrate tissues to the necessary concentration to achieve a therapeutic effect. This type of testing is carried out by pharmaceutical companies looking to patent and manufacture a new product (Gootz, 1990).

## VI. EVALUATION OF NOVEL MARINE METABOLITES

Isolated from the Sea of Japan at a depth of 289 meters (the Abyss) (Nicolau, Harrison, 2007), new strains of the marine Actinomycete genus *Verrucosispora* have yielded two new classes of cytotoxic metabolites, the proximicins and the abyssomicins. Strain MG-37 yielded three related proximicins, A, B, and C, and strain AB-18-032, now with the proposed name “*Verrucosispora maris*”, (Fiedler, et al., 2008) yielded proximicin A, B and C, and abyssomicins.

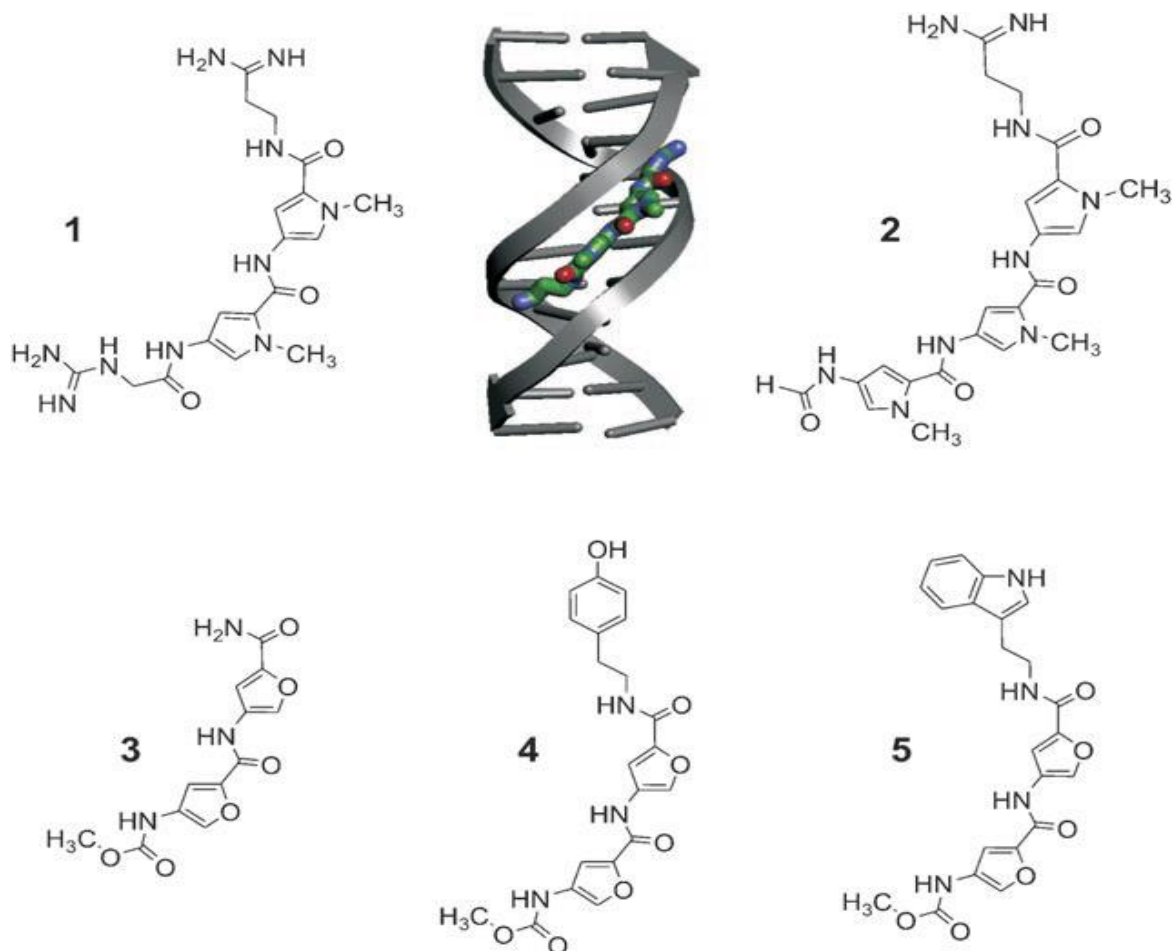
Proximicins A, B, and C are structural analogues of the antibiotics netropsin and distamycin (Schneider, et al., 2008) (Figure 5) that exhibit strong antitumor (Fiedler, et al., 2008) and moderate antibacterial activity (Brucoli, et al., 2012) (Table 1). Netropsin and distamycin inhibit DNA replication by binding directly to the minor groove of the DNA molecule but have never gained widespread use because of lack of selectivity. It was believed the proximicins would have a similar mode of action, but this was disproven by comparing the DNA denaturation curves of netropsin, distamycin, and the proximicins. Untreated DNA denatured at a mean temperature of 45°C. Due to the binding of netropsin and distamycin, the denaturation temperature is shifted higher to

78°C and 60°C, respectively. Treatment with the proximicins did not alter the denaturation temperature of DNA, indicating these molecules do not bind to DNA (Schneider, et al., 2008). To determine how the proximicins affect cell division, cell cycle analysis was performed. Gastric adenocarcinoma (AGS) cells were stained with propidium iodide and analyzed using a cell counter to obtain a baseline percentage of cells in each phase of the cell cycle. AGS cells were then incubated with netropsin, distamycin, or proximicin C, and analyzed as above to determine any alteration in the cell cycle. While both netropsin and distamycin caused an accumulation of cells in the G2/M phase, proximicin C produced an accumulation of cells in G1. This data led to an evaluation of cell cycle regulatory proteins for the transition of G1 to S phase, cyclin E, p53, and p21. AGS cells were incubated with proximicin C and cell proteins were Western blotted, revealing an upregulation of both p53 and p21. This exact mechanism of action of the proximicins on the cell cycle remains under investigation (Schneider, et al., 2008).

**Table 1 Antimicrobial activity (MIC, µg/mL) of proximicin B and C**

<b>Organism</b>	<b>B</b>	<b>C</b>
<i>S. aureus</i> ATCC29623	8	>256
<i>S. aureus</i> EMRSA-16	8	>256
<i>S. aureus</i> SA11998	4	>256
<i>S. aureus</i> EMRSA-15	8	>128
<i>Enterococcus faecalis</i>	4	>128
<i>E. coli</i>	>128	>128

Adapted from Brucoli, 2012



**Figure 5. Structures of Netropsin(1), Distamycin(2), Proximicin A(3), Proximicin B(4), and Proximicin C(5). The double helix illustrates the binding of netropsin into DNA. Adapted from Schneider, et al., 2008**

A group of secondary metabolites from “*Verrucosipora maris*” have been named abyssomicins. To date, abyssomicins B through H have been identified, but only abyssomicin C and its isomer, atrop-abyssomicin C (Figure 6), have shown effective antibiotic activity (Bister, et al., 2004). Their mode of action is inhibition of the

tetrahydrofolate biosynthesis pathway by blocking the first step, conversion of chorismate to pABA. Two enzymes catalyze the reaction, 4-amino-4-deoxychorismate (ADC) synthase and ADC lyase (Keller, et al., 2007). Abyssomicin C mimics chorismate (Figure 7) and irreversibly binds to a cysteine near the active site of PabB, a subunit of ADC synthase. This binding is dependent upon the presence of an enone Michael acceptor, which the other abyssomicins lack. An enone, a double bonded carbon adjacent to the double bonded oxygen of a ketone, is able to accept electrons from another molecule when binding. Here the enone participates in a Michael reaction with a sulfur residue on the cysteine. (Keller, et al., 2007). Antimicrobial testing was carried out against methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus* (VRSA), *Mycobacterium bovis*, and *Mycobacterium tuberculosis*, and showed a strong inhibitory effect (Table 2). This metabolite could prove to be a very effective antibiotic since its mechanism is highly selective for prokaryotic organisms.

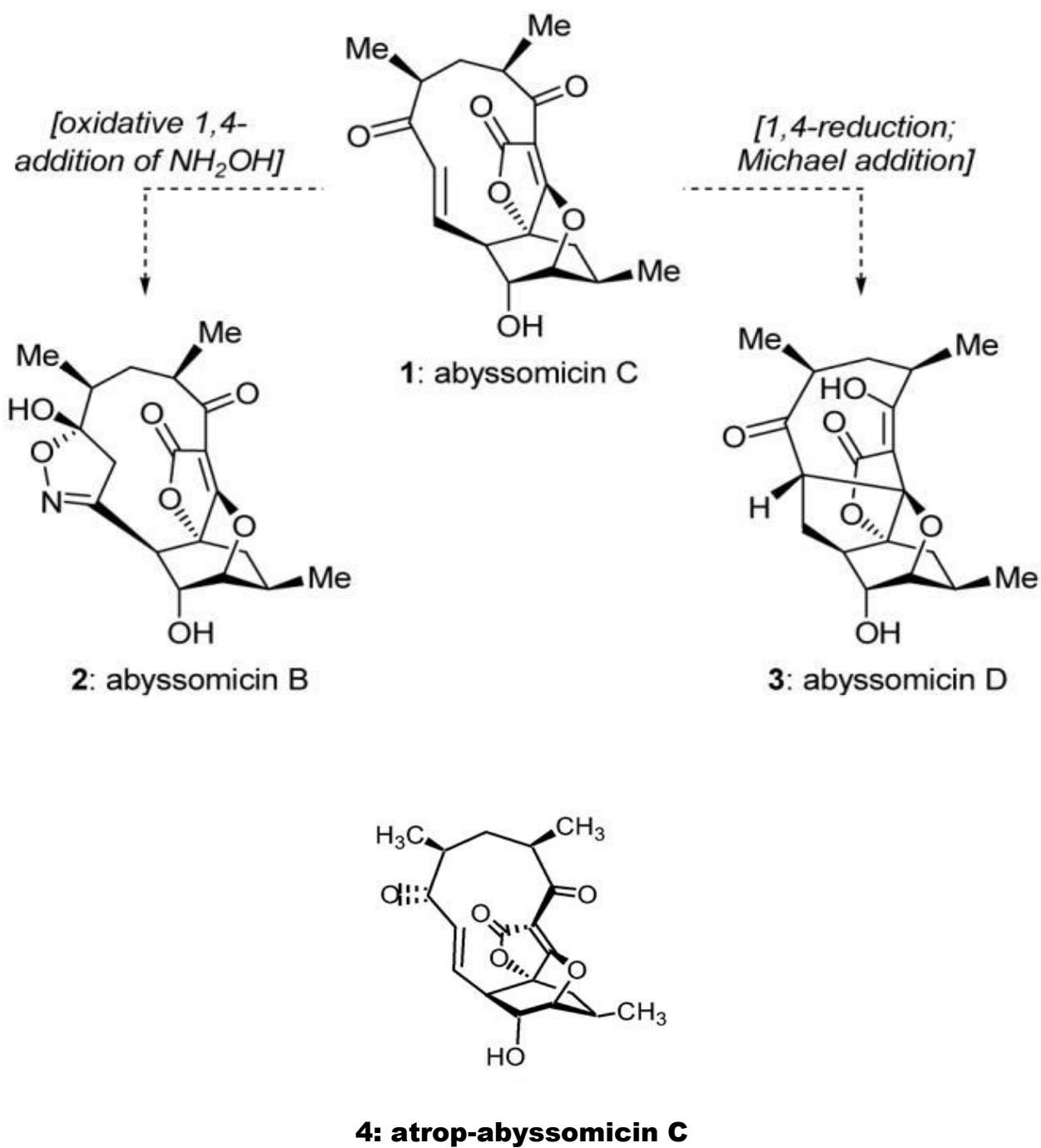


Figure 6. Structures of Abyssomicins B, C, atrop-C, and D.  
Adapted from Nicolaou, Harrison, 2007

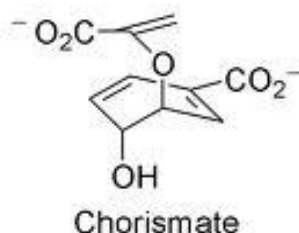


Figure 7. Structure of chorismate; compare to the structure of abyssomicins in figure 6. Adapted from Bister, et al., 2004

Table 2 Antimicrobial activity (MIC,  $\mu\text{g/mL}$ ) of abyssomicin C and atrop-abyssomicin C

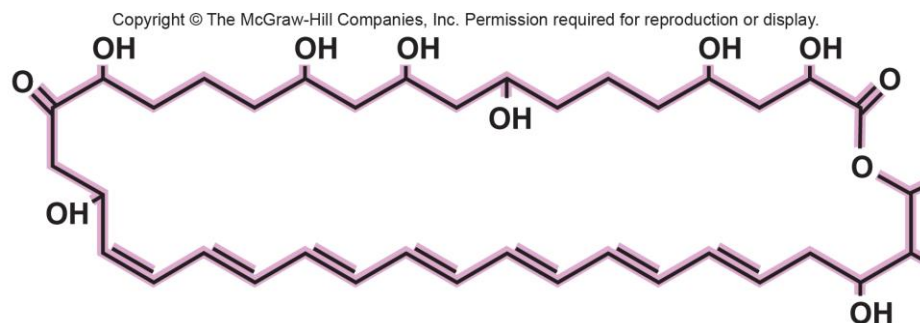
Organism	Abyssomicin C	atrop-Abyssomicin C
MRSA	4 <sup>A</sup>	3.5 <sup>B</sup>
MDR-VRSA	13 <sup>A</sup>	NT
<i>Mycobacterium smegmatis</i>	10 <sup>C</sup>	20 <sup>C</sup>
<i>Mycobacterium bovis</i>	2.5 <sup>C</sup>	5 <sup>C</sup>
<i>Mycobacterium tuberculosis</i>	1.2 <sup>C</sup>	2.5 <sup>C</sup>

Abbrev: MDR-Multi-drug resistant. NT-Not tested. Adapted from: A-from Bister, et al., 2004 B- from Nicolaou, Harrison, 2007. C- from Freundlich, et al., 2010

A marine isolate was found from a sediment sample taken from Mission Bay in San Diego. After characterization, it was determined to be unique and was suggested to be placed in a new genus with the proposed name “*Marinispora*”. The crude fermentation extract was found to be a rich source of potential new antibiotics (Kwon, et al., 2006). Since that time, more than 20 new strains of “*Marinispora*” have been identified from



diverse locations, (Kwon, et al., 2009) and several metabolites have been analyzed. Three metabolites are reported to have cytotoxic properties. The first of these metabolites was named marinomycin. The four unique isolates (A-D) are characterized as polyene macrodiolides (Kwon, et al., 2006). Macrodiolides are structurally related to the macrolide class of antibiotics but are different in function. Macrolides have ether functional groups that bind to the 50s portion of the bacterial ribosome, halting protein synthesis, where polyenes, molecules with stretches of alternating carbon double bonds, are hydrophobic molecules that integrate into the phospholipids of cell membranes; the antifungal drug amphotericin B is in this class (Figure 8). The structure of the marinomycins is a dimer composed of two identical 29 carbon subunits (Figure 9). Note the similarity to amphotericin B, with the stretches of polyene chains. With this structure, one would expect the marinomycins to act as membrane active antifungals, but they actually showed very weak activity. Marinomycin A was a potent antibiotic when tested against MRSA and VRE (Table 3) and also had impressive results in the NCI panel of 60 cancer cell lines, particularly against melanoma cell lines. The mechanism of action has not yet been determined, but would be expected to be membrane active. The large size of this molecule may also hinder its usefulness as a marketable pharmaceutical.



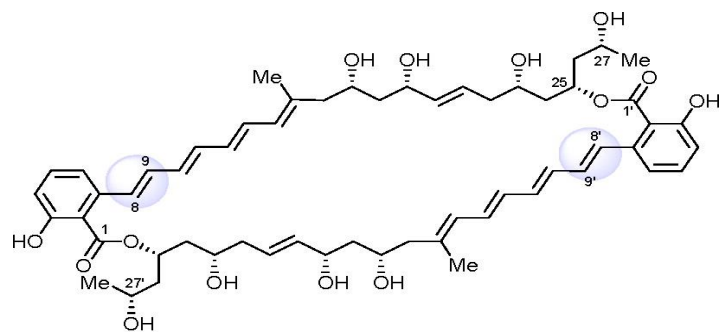
**Figure 8. Amphotericin B, a polyene.**  
Adapted from Cowan, 2012

**Table 3 Antimicrobial activity (MIC,  $\mu$ M) of Marinomycins A-D**

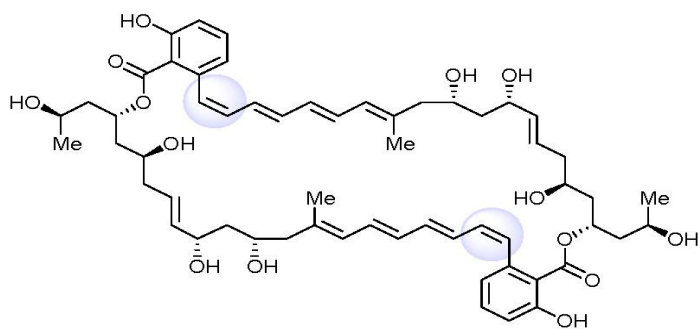
Organism	A	B	C	D
MRSA	0.13	0.25	0.25	0.25
VREF	0.13	NA	NA	NA
<i>Candida albicans</i>	7.8	NA	NA	NA

Abbrev. VREF-Vancomycin-resistant *Enterococcus faecium*, NA-no activity.

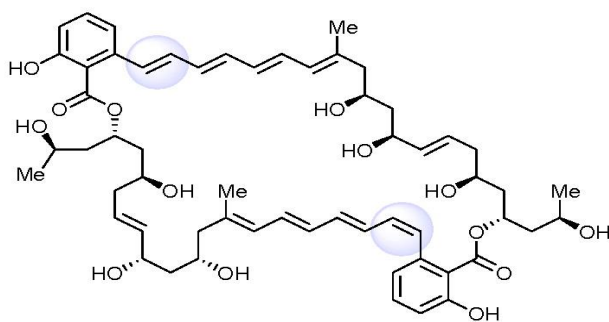
Adapted from Kwon, et al., 2006.



1: marinomycin A [*trans*  $\Delta^{8,9}$ , *trans*  $\Delta^{8',9'}$ ]



2: marinomycin B [*cis*  $\Delta^{8,9}$ , *cis*  $\Delta^{8',9'}$ ]



3: marinomycin C [*trans*  $\Delta^{8,9}$ , *cis*  $\Delta^{8',9'}$ ]

**Figure 9. Structures of Marinomycins A, B, and C.**  
Adapted from Nicolaou, et al., 2007

Also derived from "*Marinispora*" are the metabolites Lipoxazolidinones A, B, and C (Macherla, et al., 2007). They are the first natural source antibiotic of the oxazolidinone class, currently represented by linezolid (Zyvox). These block protein synthesis by binding to the peptidyltransferase center of the 70s ribosome. The active portion of these molecules is the oxazolidinone ring, a five membered ring containing nitrogen with a ketone group attached (Figure 10), which binds in such a way as to prevent transfer of *N*-formylmethionine-transfer-RNA from the A site to the P site. (Phillips, et al., 2003; Aoki, et al., 2002) The lipoxazolidinones underwent antimicrobial testing against both Gram positive and Gram negative organisms. Linezolid was also tested for comparison. The results of the testing are shown in Table 4. Lipoxazolidinones A, B, and C displayed activity at the same levels as linezolid, with effective inhibition of gram-positive organisms, and limited activity against gram-negative organisms. The large size of the molecule limits the passage through outer membrane porins.

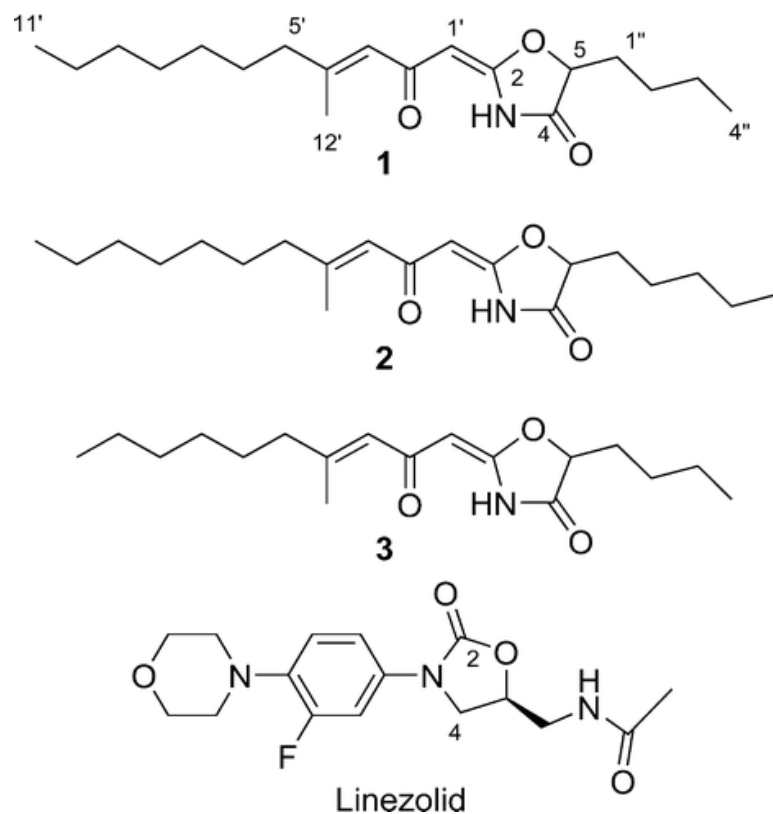


Figure 10. Structures of Lipoxazolidinones A(1), B(2), (3), and Linezolid. Adapted from Macherla, et al., 2007.

Table 4 Antimicrobial activity (MIC,  $\mu\text{g/mL}$ ) of Lipoxazolidinones A-C and Linezolid

Organism	A	B	C	Linezolid
MRSA	1	1.5	3	1.5
MDR <i>S. epidermidis</i>	0.5	0.8	NT	1
Penicillin resistant <i>S. pneumoniae</i>	4.7	6	NT	1
VREF	1.8	1.5	NT	4
<i>Haemophilus influenzae</i>	12	16	5	12
<i>Escherichia coli</i>	>32	>32	>32	>32

MDR-multi-drug resistant. NT-not tested.  
Adapted from Macherla, et al., 2007

Lynamicins A-E were isolated from *Marinispora* and identified as chlorinated bisindole pyrroles (Figure 11). Bisindole refers to the pair of double ring structures with the chlorine atoms attached, and pyrrole is the nitrogenous ring structure they are attached to. The difference between lynamicins A through E is the number of chlorine atoms on the indole groups. Lynamicin E has the fewest chlorine substitutions; it has only one. They do not fall into any previously described antibiotic class. Their mode of action remains to be found, although it appears to be linked to chlorination (McArthur, et al., 2008), since lynamicin E had the highest MIC value of the five metabolites tested. When assayed for antibiotic activity, the compounds showed potency against a number of microorganisms, including MRSA and VREF (Table 5).

**Table 5 Antimicrobial activity (MIC, µg/mL) of Lynamicins A-E**

Organism	A	B	C	D	E
MRSA	2	1	1.5	3	12
MDR <i>S. epidermidis</i>	4	1	1	4	>32
Penicillin resistant <i>S. pneumoniae</i>	24	8	20	20	>32
VREF	8	2	2	8	>24
<i>Haemophilus influenzae</i>	12	6	6	8	>32
<i>Escherichia coli</i>	16	6	6	>32	>32

MDR-multi-drug resistant.  
Adapted from McArthur, et al., 2008

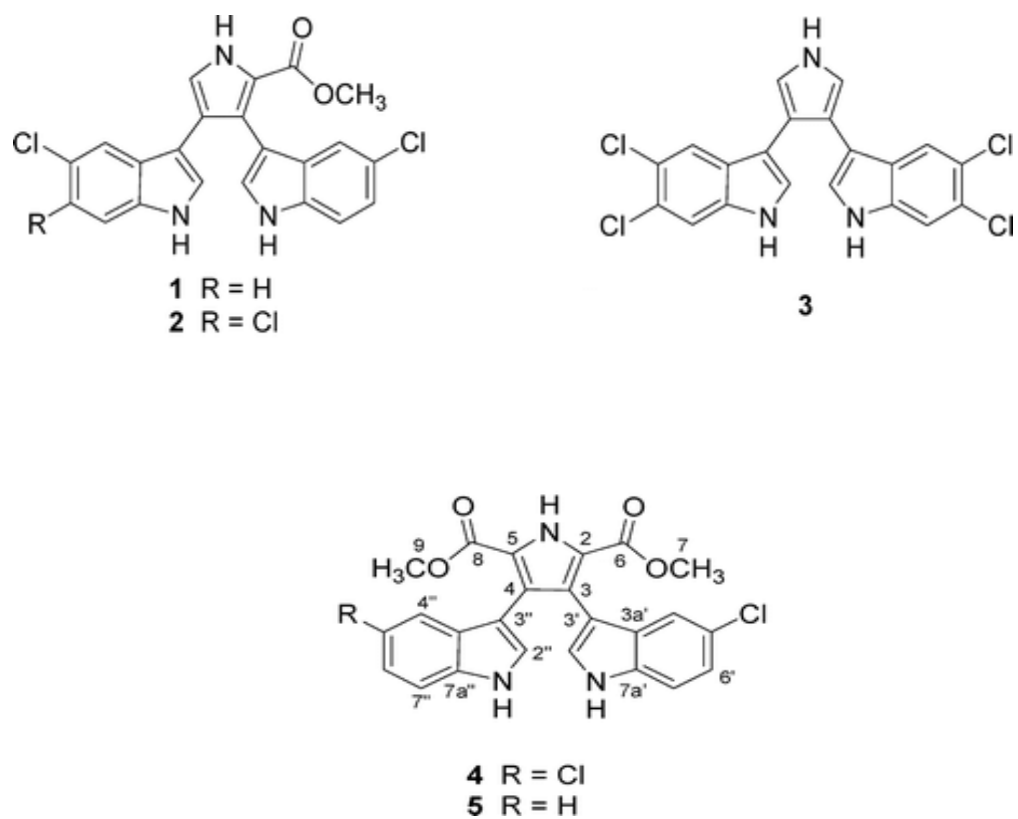
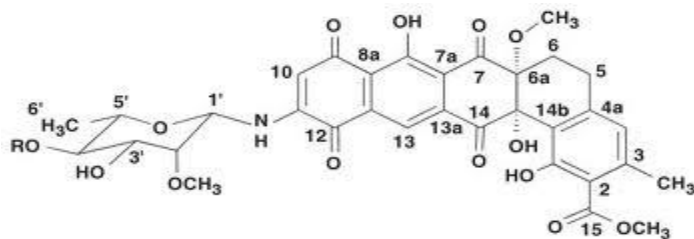


Figure 11. Lynamycins A-E, 1-5.  
Adapted from McArthur, et al., 2008

Another new group of sea water-dependant actinobacteria was discovered in marine sediments and was determined to be a unique genus that was named *Salinispora* (Jensen, et al., 2005). To date, three species have been formally described, *S. pacifica*, *S. arenicola*, and *S. tropica*. *Salinispora* species have generated much excitement as a source of secondary metabolites (Jensen, et al., 2007). Four metabolites have so far been identified from *S. pacifica*, cyanosporasides (Oh, et al., 2006), salinosporamide K (Eustaquio, et al., 2011), salinipyrones, and pacificanones (Oh, et al., 2008). All these were tested for bioactivity, but none had a cytotoxic or antibiotic effect. The six identified metabolites of *S. arenicola* have been the subject of even greater study. The arenicolides (Williams, et al., 2007), saliniketals (Williams, Asolkar, et al., 2007), cyclomarins and cyclomarazines (Schultz, et al., 2008) were not bioactive, but two effective antibiotics have been isolated, arenimycin and rifamycins B and SV. Arenimycin has been identified as a quinone derivative (Asolkar, et al., 2010)(Figure 12). Its mode of action has not been found, but it has potent antibiotic activity against gram positive pathogens, *Mycobacterium* (Table 6), and a human adenocarcinoma cell line.



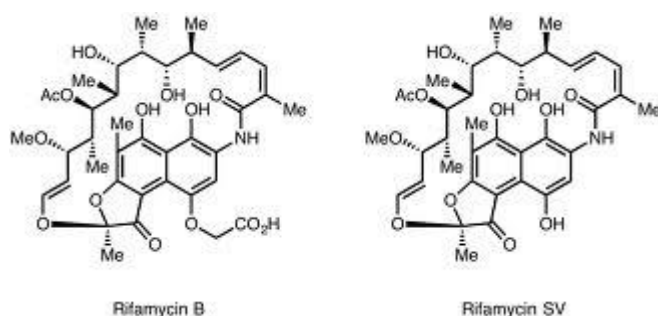


**Figure 12. Structure of arenimycin.**  
Adapted from Asolkar, et al., 2010.

**Table 6 Antimicrobial activity (MIC, µg/mL) for Arenimycin**

Organism	MIC
Rifampin and methicillin resistant <i>S. aureus</i>	1.06
MRSA (5158)	0.53
MRSA (5085)	1.03
MRSA (5167)	0.13
MRSA (5177)	0.05
MRSA (5218)	1
<i>Staphylococcus epidermidis</i>	0.05
<i>Staphylococcus saprophyticus</i>	0.1
<i>Enterococcus faecalis</i>	0.06
<i>Enterococcus faecium</i>	0.25
VREF	>8
<i>Mycobacterium bacille</i> (Calmette Guerin)	1

Adapted from Asolkar, et al., 2010

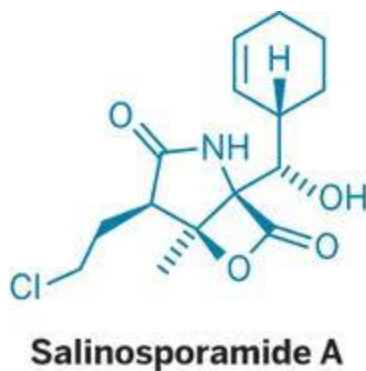


**Figure 13. Structures of the rifamycins.**  
Adapted from Kim, et al., 2006.

Rifamycins B and SV (Figure 13) are known antibiotics from the soil bacteria *Amycolatopsis mediterranei* and have been the basis for antibiotics in the rifampin family. The discovery of the rifamycin gene clusters in the unrelated species *S. arenicola* provides a new source for this important antibiotic (Kim, et al., 2006). Rifamycin binds to bacterial RNA polymerase molecules and prevents the lengthening of the mRNA chain. Because of their size, they are only useful for gram positive organisms, as they are too large to pass through the porins of the gram negative membrane.

None of the metabolites from *Salinispora tropica* displayed antibiotic activity (Jensen, et al., 2007), however, salinosporamide A (Figure 14) has potent cytotoxic activity, and, under the trade name Marizomib, is currently in clinical trials for the treatment of many cancers, including multiple myeloma, lymphoma, leukemia, glioma, colon, and

pancreatic (Potts, et al., 2011). Marizomib is an inhibitor of the 20s subunit of the proteasome (Williams, et al., 2005). Because of this strong cytotoxic effect, salinosporamide A was tested and found to be effective against *Plasmodium falciparum*, the causative agent of malaria (Prudhomme, et al., 2008), and *Trypanosoma brucei*, the causative agent of African sleeping sickness (Steverding, et al., 2012).



**Figure 14 Structure of Salinosporamide A.**  
Adapted from Jensen, et al., 2007

## VII. THE NEXT STEPS

The compounds discussed here are in the earliest stages of analysis. The inhibition of microbial growth in microtiter wells is the first step of a very long process to bring new drugs to market (reviewed by Gootz, 1990). A pharmaceutical developer will evaluate the potential marketability of a compound based on what is known about the structure and mechanism of action. Then culture and purification systems must be optimized to provide the large quantities of compounds needed for testing. Analysis then moves from *in vitro* to *in vivo*, starting with administration to rodents. A compound must undergo studies that characterize its mechanism of action and extensively assess its safety and efficacy in animals. A new metabolite is rarely marketed in its original form due to deficits in activity or potency, *in vivo* stability or pharmacokinetics, or an unacceptable safety profile as determined in animal studies. Many metabolites are manipulated chemically to assess the changes in biological activity. These are called structure-activity relationships (SAR). Chemical substitutions around the biological active site can improve the deficits mentioned above, the safety profile and prevent the activity of resistance mechanisms. All the various pharmacokinetic variables for each compound are tested. Additional animal studies predict the antibiotic's effects in humans and are

then used to determine how well the agent will cure an infection. These are called in vivo protection studies and they help determine the proper effective dose that protects 50% of the animals from death due to infection (PD<sub>50</sub>). Toxicity studies are also included to assess acute and chronic dosing schedules with regard to safety, reproductive effects, organ dysfunction (especially liver and kidney), blood abnormalities, carcinogenicity, behavioral changes and lethality. There are several factors that can limit the results of animal testing. Some of these factors are practical in nature. Animal testing is more expensive, and is subject to increased ethics scrutiny, which keeps the numbers of trials at the bare minimum needed for proper data collection. Time is another practical constraint; the shorter lifespan of small mammals limits true long-term toxicology and carcinogenicity studies. Other factors are biological in nature. Small mammals generally metabolize faster, allowing them to breakdown and eliminate drugs more rapidly. There are also different permeability rates based on different cell transport mechanisms. To make accurate predictions despite these differences, researchers rely on animal models, established protocols that are based on animal and human comparative data derived from years of previous research of similar drugs (Gootz, 1990). Researchers must choose which animal model will be the best fit for their drug.

Successful safety studies allow the drug to be considered for clinical trials, and the Food and Drug Administration (FDA) website details all the requirements for this process. Every pharmacological variable is carefully detailed for the first step, the investigational

new drug (IND) application. The IND must contain data in three general areas, animal pharmacological and toxicology studies as discussed above, manufacturing information, to ensure the production company can adequately and safely produce enough drug for trials, and clinical protocols and investigator information to determine the planned studies, the risks involved, and to ascertain the qualification of the investigator (<http://www.fda.gov/drugs/developmentapprovalprocess/howdrugsaredevelopedandapproved/approvalapplications/investigationalnewdrugINDapplication/default.html>). After the IND is filed, the developer must wait 30 days before starting trials to allow the FDA to review the application.

The antimicrobial then begins Phase 1 investigation, which involves the first administration to humans. The total number of healthy volunteers required is approximately 100. The goal of Phase 1 is not to determine effectiveness, but to establish metabolic and pharmacologic action in humans along with possible toxicological effects. The generated data are used to design effective Phase 2 studies. Phase 2 studies are controlled studies to evaluate the effectiveness of the drug and to determine the short term side effects. The several hundred subjects are individuals whose infections are non-life threatening. Phase 3 trials are performed after preliminary effectiveness is determined in Phase 2 and are designed to gather additional information about effectiveness, safety and dosage. This testing requires several years and several thousand subjects.

Following the completion of Phase 3 studies, the pharmaceutical company must organize all its data and submit the New Drug Application (NDA) to the FDA for review. The NDA will encompass information on manufacturing specifications, stability and bioavailability data, method of analysis of dosage forms, packaging and labeling and any additional toxicology studies (<http://www.fda.gov/drugs/developmentapprovalprocess/howdrugsaredevelopedandapproved/approvalapplications/newdrugapplicationNDA/default.html>). The review takes from 18 months to two years to complete, and then the antibiotic is permitted to be marketed in the United States.

Since microbes will always find new resistance mechanisms, humans must continue to hunt for new antibiotics. The marine Actinobacteria are an untapped source for these new compounds, and will hopefully yield some marketable pharmaceuticals.

Undoubtedly, there are countless microbial species just waiting to be discovered within the vast oceans, and current sampling techniques only identify a small fraction. As we continue to refine molecular techniques, such as 16S rRNA identification, our discoveries will increase. Additionally, genomic analysis can lead to the discovery of biosynthetic pathways for even more metabolites than can be found through culture techniques and can give clues as to the growth conditions required for the production of said metabolites. Isolation of such genetic sequences can also allow them to be utilized in recombinant technology to mass-produce large quantities.

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